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Low density Porcicoll separates spermatozoa from bacteria and retains sperm quality

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ABSTRACT

Antibiotics are added to semen extenders to control the growth of bacteria contaminating semen during collection but may contribute to the development of antibiotic resistance. An alternative would be physical separation of spermatozoa from bacteria. The objective of the present study was to evaluate two low densities of Porcicoll for removal of bacteria, and for their effect on sperm recovery and sperm quality. Semen was collected from boars at a commercial station. Aliquots of 8 extended ejaculates were subjected to colloid centrifugation through 20% Porcicoll (P20) and 30% Porcicoll (P30) in 500 mL tubes and then stored at 17 °C. Microbiological examination and sperm quality evaluation (computer assisted sperm analysis and flow cytometry) were carried out on controls and all colloid-selected samples immediately after preparation and again after storage for 3 and 7 days. The microorganisms found were mainly bacteria from the environment, gut or skin. There was a considerable reduction or complete removal of some bacteria by both colloids. Recovery rates were 86% for P20 and 81% for P30. Sperm quality was not adversely affected by colloid centrifugation on day 0, and thereafter showed a more gradual deterioration in colloid centrifuged samples than in controls, possibly due to lower bacterial contamination. There were no differences in sperm quality between the two colloid treatments. Thus, these results show that contaminating bacteria in semen can be controlled by centrifugation through low density colloids.

1. Introduction

Antimicrobial resistance is a major global challenge in both human and veterinary medicine; it was reported by the World Health Organisation (WHO) to be the biggest threat to the population of the modern world [1]. Genes for antimicrobial resistance are passed readily between different bacteria [2], so that even limited antibiotic usage can lead to considerable resistance developing [3]. For this reason, prudent use of antibiotics is advocated i.e. antibiotics should be used only when strictly necessary, for therapeutic purposes, and after testing the bacteria for sensitivity to the proposed therapeutic agent.

One area of concern is in artificial insemination (AI) where extenders for preparing semen doses contain antibiotics. Bacteria colonise the mucosa of the distal part of the reproductive tract from the animal's skin and its environment and semen becomes contaminated during ejaculation. Direct contamination from the environment is also possible despite standardized hygiene protocols [4]. Bacterial contamination is of particular relevance in swine AI where the majority of inseminations are carried out with liquid semen rather than with frozen semen [4]. Bacteria can proliferate at the usual storage temperatures for boar semen (16–18 °C) [5], competing with spermatozoa for nutrients. They also have a detrimental effect on spermatozoa, producing metabolic byproducts and toxins, and may even cause infection and infertility in the sow after insemination, or result in the spread of

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infection [6]. Therefore, international regulations [7] mandate the addition of antibiotics to semen extenders. However, resistance to these antibiotics has been detected in bacteria found in semen [4,8], requiring the use of additional antibiotics [8].

An alternative to adding antibiotics to semen extenders could be to remove bacteria from semen by physical means. Centrifugation through a single layer of colloid (SLC) of density 1.104 g/mL, separated spermatozoa from bacteria in boar semen when performed immediately after semen collection, and reduced the number of bacteria if performed several hours after semen collection [9]. However, this technique selects robust spermatozoa from the rest of the ejaculate [10]; therefore, part of the sperm population is lost. This sperm loss, together with the cost of the colloid, resulted in reluctance from the pig industry to adopt this method as an alternative to antibiotics. Recently, a low density colloid (1.052 g/mL) which allows the recovery of most of the spermatozoa, was successful in separating spermatozoa from seminal plasma without selecting for good quality spermatozoa [11]. Sperm quality remained good during one week's storage, despite the lack of selection to remove poor-quality spermatozoa [11].

A pilot study was conducted to determine whether even lower densities of colloid could be used in 50 mL centrifuge tubes without impinging on sperm quality. In this case, both 20% and 30% Porcicoll were tested (densities 1.026 g/mL and 1.039 g/mL, respectively), recovering almost all of the spermatozoa, and sperm quality remained high during storage for one week [12]. However, since the commercial semen doses used in the study by Deori et al. contained antibiotics, it was not possible to test the effectiveness of separating spermatozoa from bacteria. The present study, therefore, was designed to test 20% and 30% Porcicoll for their ability to separate spermatozoa from bacteria, using scaled-up SLC to process 200 mL of extended semen in 500 mL tubes.

2. Methods

2.1. Experimental design

Semen was collected at a commercial pig breeding company, following a less strict hygienic protocol than usual in order to obtain the type of bacterial contamination that could occur accidentally in practice. The intention was to ensure that some bacteria were present, in order to test the ability of the colloid to remove them. The ejaculates were extended in extender without antibiotics. Part of the ejaculate was prepared by SLC with low density colloids, while the remainder served as a control. Samples were subsequently stored at 17 °C. Sperm quality in all samples was evaluated by computer assisted sperm motility analysis (CASA) and flow cytometry immediately after the sample preparation was completed (day 0), as well as on day 3 and day 7.

2.2. Semen extender

Modified Beltsville Thawing Solution (BTS) [10] was used as extender. The BTS was prepared in the laboratory without antibiotics and consisted of glucose (205.4 mM), tri-sodium citrate (20.4 mM), sodium hydrogen carbonate (14.9 mM), sodium EDTA (3.4 mM) and potassium chloride (10.1 mM).

2.3. Animals and semen collection

Ten boars (1 Duroc, 2 Landrace, 1 York, 5 Large White and 1 Pietrain) were kept at a commercial pig station (Technological Centre of Artificial Insemination; Topigs-Norsvin Spain, Campo de Villavidel, León, Spain) under standard husbandry conditions, according to national and international regulations on the housing and care of animals. Boars (12–35 months of age) were kept in individual pens in a controlled environment of 18–22 °C and constant photoperiod 12 h/d, with 2.5 kg/d of a 13% protein diet and water ad libitum. Since the animals are kept for the purpose of semen collection (twice weekly), and no experimental procedures were carried out on the animals themselves, no ethical permission was required for this study.

Semen was collected using the gloved hand technique [13]. Sperm concentration, subjective motility and normal morphology were assessed, while the ejaculates were kept in a water bath at 32.5 °C. Only normospermic ejaculates with acceptable quality (≥80% motile spermatozoa, ≥75% morphologically normal and ≥95% normal acrosomes) were used for this study. Ejaculates were then extended with antibiotic-free BTS at 32.5 °C to the concentration required for the study (100×10^6 spermatozoa/mL) with a dilution factor of 2.4 ± 0.5. Since 450 mL of extended semen was required for the experiment, four ejaculates of low volume were pooled in pairs to form two samples, resulting in a total of 8 samples for the study. These samples were transported to the semen processing laboratory at the Institute of Animal Health and Cattle Development (INDEGSAL), University of León, Spain, (approximately 20 min) in an insulated container to maintain a temperature of 18 °C during transport.

2.4. Sample preparation

The colloids used were modifications of a silane-coated silica formulation for boar semen (Porcicoll), prepared at a density of 1.039 g/mL and 1.026 g/mL for 30% Porcicoll (P30) and 20% Porcicoll (P20), respectively. Each extended semen sample was split into three portions: uncentrifuged control (CON), SLC with P30 and SLC with P20. The SLC samples were prepared in 500 mL tubes [14] using 150 mL of the low density Porcicoll and 200 mL extended semen, centrifuged at 300×*g* for 20 min. After centrifugation, the supernatant was removed using a water pump and the sperm pellet was resuspended in sterile BTS up to 100×10^6 spermatozoa/mL, as in our previous study [11]. The final volume of the resuspended sperm pellet was equivalent to the original volume of semen loaded on the colloid. The sperm suspensions were transferred to 15 mL tubes for storage at 17 °C. An aliquot (0.5 mL) of each sample was immediately transferred to the microbiology department on ice.

2.5. Sperm concentration and morphology

Sperm concentration was assessed with a haemocytometer (Bürker chamber) in duplicate, counting at least 400 cells. Recovery rate (%) was calculated from the following formula:

Recovery rate = (no. spermatozoa in sperm pellet/no. spermatozoa loaded on colloid)*100.

An aliquot of each sample was fixed in PBS with 0.5% formaldehyde and kept at 5 °C. For cell morphology, a 5-μl drop was observed at × 400 with negative contrast optics (Nikon E400 microscope), assessing at least 200 cells. The proportion of spermatozoa with abnormalities was recorded, classifying abnormalities according to their location i.e. head, midpiece and principal piece, and the presence of a cytoplasmic droplet.

2.6. Microbiological and MALDI-TOF analyses

Procedures were carried out as previously described [11], following validated protocols [15]. Briefly, the samples (0.1 mL) were cultured for 24 h at 37 °C in a microaerophilic atmosphere on various media: Blood Columbia agar, Cystine-Lactose-Electrolyte-Deficient (Cled) agar, McConkey agar and tryptone soy agar (TSA; OXOID, Hampshire, UK).

For the bacterial count, dilutions were made from −1 to −6, followed by seeding 100 μL in Agar TSA. The number of colonies was counted (colony forming units/mL; CFU/mL). Plates were read again after a further 24 h incubation.

Bacteria were characterized using different methods depending on type: Gram stain, oxidase and catalase activity and different biochemical test (API 20E, API 20NE, API Staph, API Strep; Bio Merieux Inc., Durham, NC), according to the manufacturer's instructions.

Samples were analysed using a Bruker Daltonics UltrafleXtreme MALDI-TOF/TOF equipment and the FlexControl software v. 3.0 (Brucker Daltonics, Bremen, Germany) for the acquisition of mass spectra. The Biotyper Real Time Classification software v3.1 (Brucker Daltonics) was used for microbial identification by comparison of the spectra with the corresponding database provided by the manufacturer (MALDI Biotyper database, 5989 entries, Bruker Daltonics). This software generates a score, ranging from 0 to 3, showing the similarity between a given sample and a reference spectrum, and displays the top 10 matching results with the highest scores. The reliability of the identification was evaluated according to standard interpretative criteria: 2.300–3.000, high species identification probability; 2.000–2.290, high genus identification probability; 1700–1.999, presumable species identification; 1.700–1.999 presumable genus identification; 0.000–1.699 unreliable identification.

2.7. Sperm analyses

2.7.1. Motility analysis by CASA

A 5 μL drop was pipetted into a modified Makler counting chamber (20 μm depth; Haifa Instruments, Israel) and evaluated with a phase contrast microscope (Nikon E400 with warmed stage at 37 °C; 10x negative contrast optics) provided with a Basler A312f camera (Basler AG, Ahrensburg, Germany). Image sequences were acquired from at least three independent fields at 53 frames/s (at least 200 motile spermatozoa per sample, except in those with extremely low motility). Image sequences were analysed with the ISAS 1.0.18 software (Proiser SL, Valencia, Spain), for total (MOT %; VAP>10 μm/s) and progressive motility (PROG, %; VAP>25 μm/s and STR >45%)) for each sample, and the following kinematics for each motile spermatozoon: VCL (curvilinear velocity; μm), VSL (straight path velocity; μm), VAP (average path velocity according to the average smoothed path; μm/s), LIN (linearity; %), STR (straightness; %), WOB (wobble; %), ALH (amplitude of the lateral head displacement, μm), BCF (beat cross frequency, Hz) [16].

2.7.2. Flow cytometry

Combinations of fluorescent probes in PBS (0.5% BSA) were used for evaluating various sperm properties [17,18]. Hoechst 33342 (H342, 4.5 μM) was used to facilitate discrimination of debris; Hoechst 33258 (H258, 4.5 μM) or propidium iodide (PI; 1.5 μM) for viability; YO-PRO-1 (100 nM) for apoptotic-like changes; PNA-FITC (PNA; 1 μg/ml) for assessing the acrosomal status; H_2DCFDA (CFDA; 5 μM) for detecting cytoplasmic reactive oxygen species (ROS); merocyanine 540 (MC; 2 μM) for assessing capacitation-like changes; MitoSOX (MX; 1 μM) for detecting mitochondria-produced superoxide; Mitotracker Deep red (MT; 100 nM) for assessing mitochondrial activity. These probes were combined as H342/YO-PRO-1/M540/PI/MT (viability, apoptosis, capacitation, mitochondrial activity), H342/PNA/PI (viability and acrosomal status) and H258/CFDA/MitoSOX (viability, cytoplasmic ROS and mitochondrial superoxide). After adding the spermatozoa (10^6/mL), the mixture was incubated in the dark for 15 min at 37 °C.

The data were analysed using Weasel v3.4 [19] (http://www.frankbattye.com.au), obtaining the proportion of acrosome-damaged spermatozoa (total PNA^+); the proportion of acrosome-damaged spermatozoa in the live population (PNA^+ within PI^-); the proportion of YO-PRO-1^- spermatozoa as viable-non apoptotic; the ratio of non-apoptotic within viable (YO-PRO-1^+ within the PI^- population); the ratio of capacitated within viable-non apoptotic ($M540^+$ within the YO-PRO-1^- population); the proportion of spermatozoa with active mitochondria as YO-PRO-1^-/MT^+; the ratio of cytoplasmic ROS production within the viable population ($CFDA^+$ within the $H258^-$ population); and the ratio of high mitochondrial-superoxide production within the viable population ($MitoSOX^+$ within the $H258^-$ population).

Chromatin status was assessed using the Sperm Chromatin Structure Assay (SCSA), as described previously [18]. Briefly, samples were stored at −80 °C in TNE (2×10^6/mL; 0.01 M Tris-HCl, 0.15 M NaCl, 1 mM Na_2EDTA; pH 7.4). After thawing, 200 μL of sample were mixed with 0.4 mL of acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100; pH 1.2). After 30 s, 1.2 mL of staining acridine orange (AO) solution (6 μg/mL AO in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM Na_2EDTA, 0.15 M NaCl; pH 6.0) were added, incubating for 3 min. The samples were run through a FACScalibur flow cytometer with the acquisition software CellQuest v. 3 (Becton Dickinson, Franklin Lakes, NJ, USA), acquiring at 200 cells/s and at least 5 000 spermatozoa; AO was excited with the Ar-ion 488 nm laser, detecting green fluorescence with a 530/30 filter (dsDNA-bound AO), and the red fluorescence with a 650 long-pass filter (ssDNA-bound AO). Data were saved in flow cytometry standard (FCS) v. 2 files, and processed using the R statistical environment (R Core Team 2019) to obtain the proportion of DNA-damaged spermatozoa (%DFI).

2.8. Statistical analysis

Data were analysed using linear mixed-effects models (R statistical environment) [19] with treatments and storage time as fixed effects. Pairwise comparisons were adjusted by Tukey's method. Results are presented as means ± SEM; the threshold for significance was set at $P < 0.05$. The analysis of associations between bacterial load and sperm quality was carried out by Spearman correlation, with $P < 0.01$ being considered significant, accounting for type I errors.

3. Results

3.1. Recovery rate

The mean (±SD) recovery rate after SLC was 85.9 ± 10.5% for P20 and 80.7 ± 13.7% for P30.

3.2. Bacterial content

The bacterial counts for all samples are summarized in Table 1. The main bacterium identified was Aeromonas caviae, accounting for 50% of the total bacteria in control samples at time 0. There were significant associations between most of the bacterial species (Fig. 1).

The bacterial count in controls increased considerably during storage (Table 1). The bacterial count was reduced in the SLC samples; only A caviae was present above 100 cfu/mL in P30 samples on day 0, whereas small numbers of E. coli in addition to A. caviae were present in P20 samples (Table 2). The bacterial counts increased with time in all groups, with more species and greater numbers of bacteria being found on days 3 and 7 than on day 0. However, the bacterial counts were lower for SLC than controls on day 3 ($p < 0.05$) and day 7 ($p < 0.01$), mostly being A. caviae. The majority of the SLC samples were negative for bacteria even on day

Table 1
Total bacterial counts for each boar (× 10^3 CFU/mL), excluding Aeromonas, in control and samples after Single Layer Centrifugation through 20% Porcicoll (P20) and 30% Porcicoll (P30).

	day 0			day 3			day 7		
Sample	CON	P20	P30	CON	P20	P30	CON	P20	P30
1	0.2	<0.1	<0.1	>1000	0.2	<0.1	>1000	<0.1	<0.1
2	0.5	<0.1	<0.1	0.7	0.2	0.2	34.3	<0.1	<0.1
3	6.7	0.2	0.2	>1000	0.7	0.2	>1000	>1000	4.2
4	132.4	132.2	0.2	>1000	>1000	<0.1	>1000	>1000	0.2
5	11.9	0.2	0.2	32.2	0.2	0.2	118	0.2	0.2
6	<0.1	<0.1	<0.1	1	<0.1	<0.1	2.3	<0.1	<0.1
7	0.8	<0.1	<0.1	0.6	0.6	<0.1	6.3	3.3	<0.1
8	7.7	1	<0.1	34.8	<0.1	<0.1	>1000	<0.1	<0.1
Median	3.75	0.15	<0.1	33.5	0.2	<0.1	>1000	0.15	<0.1
Quartiles	0.425, 8.75	<0.1, 0.4	<0.1, 0.2	0.925, >1000	0.175, 0.625	<0.1, 0.2	27.3, >1000	<0.1, 252.475	<0.1, 0.2

7 (Table 3).

3.3. Sperm quality

3.3.1. Sperm morphology

The proportion of spermatozoa with abnormal heads was decreased in the SLC samples compared to controls (controls 4.8% ± 0.4, P20 2.5% ± 0.3, P30 2.7% ± 0.3; $p < 0.001$). The proportion with proximal cytoplasmic droplets was not different between groups (controls 7.5% ± 0.6, P20 7.3% ± 0.6, P30 6.2% ± 0.2; NS). Midpiece defects (controls 5.6% ± 0.9, P20 6.6% ± 0.7, P30 7.1% ± 0.6) and principal piece defects (controls 1.1% ± 0.3, P20 0.9% ± 0.3, P30 0.8% ± 0.1) were also not different between groups. However, detached heads were more prevalent in the P30 group than in the other groups (control 0.5% ± 0.2, P20 0.19% ± 0.2, P30 1.2% ± 0.3; $p < 0.025$). There was significant inter-individual variation for all abnormalities ($p < 0.001$).

3.3.2. Motility results (CASA and subpopulation analyses)

Although total and progressive motilities were slightly decreased for the SLC samples compared to controls on day 0 (Fig. 2), they recovered on day 3 and 7 when they were higher than controls ($p < 0.001$). This effect on day 0 was, at least in part, due to spermatozoa adhering to the motility chamber, as previously observed with SLC of boar sperm samples. There was a significant treatment*time interaction ($p < 0.004$) for these variables, as well as for linearity-related variables, because sperm quality in control samples deteriorated more rapidly than SLC samples with time. The kinematics VCL, VSL and VAP were not different among treatments, although there was a trend for VAP to differ among time points ($p = 0.055$). The ratios LIN, STR and WOB showed treatment*day interactions ($p = 0.02$, $p = 0.046$ and $p = 0.028$, respectively). Overall, BCF was higher in SLC samples ($p = 0.008$) whereas ALH was not significantly affected by treatment.

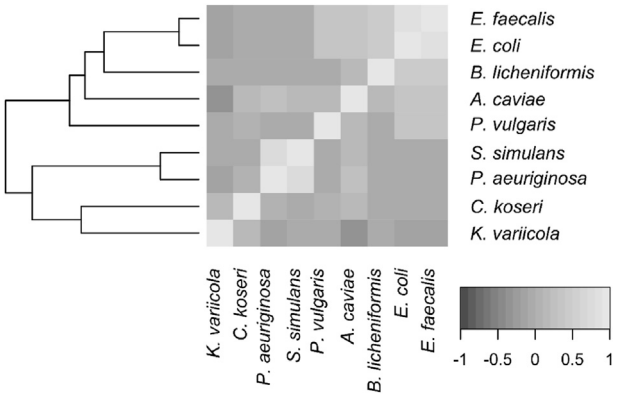

Fig. 1. Correlation matrix (heatmap) and dendrogram for associations between the bacterial species found in boar semen samples (closely related species show shorter distances before joining). The key shows the strength of the correlations (r value).

3.3.3. Flow cytometric analysis

In most cases, sperm quality in SLC and Control samples was similar at day 0 (Fig. 3), followed by a more rapid deterioration with time for Controls than SLC. Thus, viability (Fig. 3a) and mitochondrial activity (Fig. 3h) decreased with time in the three groups whereas apoptotic ratio (Fig. 3b) and acrosomal damage (Fig. 3c) increased ($p < 0.001$). In all cases, the Control samples changed at a greater rate (treatment*time interaction, $p < 0.05$ at day 7). For damaged acrosomes within the viable population (Fig. 3d), values for SLC samples did not change during storage whereas those for Control samples increased considerably ($p = 0.016$). The two SLC treatments maintained similar values during storage, except for the ratio of capacitated spermatozoa within the viable population (merocyanine staining), with P30 showing significantly lower values than Controls ($p < 0.013$), whereas P20 values were not significantly different to Controls.

The ratios of viable spermatozoa positive for cytoplasmic mitochondrial ROS (Fig. 3g), and the DNA fragmentation (Fig. 3i) did not differ between treatments, although they increased with storage time ($p < 0.001$).

3.4. Associations between specific bacteria and sperm quality

Sperm quality was adversely affected by the presence of specific bacteria (Table 4). In most cases the presence of bacteria was positively correlated to apoptotic ratio, damaged acrosomes, ROS production, and DNA damage, and negatively correlated to viability. A. caviae and E. faecalis affected many parameters of sperm quality, notably those associated with mitochondrial status and ROS production, acrosomal status, viability and DNA damage. Only A. caviae was negatively associated with total and progressive motility. Of the less abundant species, K. variicola, was associated mostly with motility variables and the apoptosis and capacitation ratios, whereas C. koseri only affected acrosomal integrity. P. aeruginosa and the other species were not strongly ($P < 0.01$) associated to sperm quality variables. The total CFU/mL excluding Aeromonas, was negatively associated with sperm viability, and positively associated with acrosomal status, mitochondrial ROS production and DNA damage.

4. Discussion

The purpose of this study was to determine whether 20% or 30%

Table 2
Median and quartiles for each bacterial species ($\times 10^3$ CFU/mL) in control and samples after Single Layer Centrifugation through 20% Porcicoll and 30% Porcicoll at three time points.

	day 0			day 3			day 7		
Species	CON	P20	P30	CON	P20	P30	CON	P20	P30
A. caviae	4.9(2.3, 19)	6.2(1.5, 26.2)	5.9(2.7, 27.2)	536.5 (8.4, 1000)	32.2(1.8, 1000)	51.8 (3.1, 1000)	1000 (1000, 1000)	1000 (750.9, 1000)	1000 (752, 1000)
P. aeuriginosa	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.1 (0, 6.5)	0 (0, 0.1)	0 (0, 0.1)	1 (0, 13.7)	0 (0, 0.1)	0 (0, 0.1)
S. simulans	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0.3)	0 (0, 0)	0 (0, 0)	0 (0, 3.9)	0 (0, 0)	0 (0, 0)
E. coli	0.6 (0, 6.1)	0 (0, 0.3)	00, 0.1)	0.4 (0, 251.9)	0 (0, 0)	0 (0, 0)	15.7 (0, 1000)	0 (0, 0)	0> (0, 0)
E. faecalis	0 (0, 0.6)	0 (0, 0)	0 (0, 0)	0> (0, 33.2)	0 (0, 0.2)	0 (0, 0)	0 (0, 1000)	0 (0, 250)	0 (0, 0)
B. licheniformis	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0.6)	0 (0, 0)	0 (0, 0)
C. koseri	0 (0, 0)	0 (0, 0)	0 (0, 0)	0 (0, 0.7)	0 (0, 0)	0 (0, 0)	0 (0, 1)	0 (0, 0)	0 (0, 0)
K. variicola	0 (0, 0.1)	0 (0, 0.1)	0 (0, 0.1)	0 (0, 0.1)	0 (0, 0.1)	0 (0, 0)	0 (0, 0.1)	0 (0, 0.1)	0 (0, 0.1)

P. vulgaris showed 0 (0, 0) in all cases; it was only detected in one Control (CON) sample. P20 and P30 refer to 20% and 30% Porcicoll, respectively.

Porcicoll in 500 mL tubes could be used to separate spermatozoa from most of the bacteria in boar semen, without an adverse effect on sperm quality. The results showed that both P20 and P30 could be used to remove most of the bacteria, apart from A. caviae, and that SLC did not have an adverse effect on sperm quality. In fact, except for lower total and progressive motility immediately after SLC, sperm quality was not affected by processing. On the contrary, deterioration in sperm quality was slower in the SLC samples than in the controls, so that by day 7 sperm quality was significantly better in SLC samples than in controls.

The recovery rates in 500 mL tubes, 86% for P20 and 80% for P30, were lower than in 50-mL tubes, where the recovery rates were 94 ± 18% and 87 ± 15%, respectively [12]. It is not known why recovery rate should be lower when the SLC was done in 500-mL tubes than in 50-mL tubes used in the previous studies [11,12], but the sample size in both cases was small (8 and 10 samples, respectively); any differences may be less apparent with a larger sample size. The lower motility immediately after SLC was assumed to be due to spermatozoa adhering to surfaces or to each other. This effect was observed in previous experiments [11], and tends to resolve during storage, although it can make motility evaluation problematic.

Sperm quality on day 7 in the present study was not as high as in a previous study using P40 in 50 mL tubes [12], possibly due to the relatively higher bacterial loads throughout the present study, especially A. caviae. Thus, membrane integrity in the previous study was 73–75% in SLC samples compared to 70% in the present study, damaged or reacted acrosomes was approximately 6% compared to 22%, and the proportion of spermatozoa with active mitochondria was 75% compared to 60%. However, the proportion of capacitated spermatozoa was 9% in the previous study, compared to 4–6% in the present study. Sperm quality in control samples was lower than SLC samples by day 7 in the present study (membrane integrity 55% versus 72%, acrosome reacted 50% compared to 16%, capacitated spermatozoa 6% compared to 3.2%, and the proportion of spermatozoa with active mitochondria was 45% compared to 73%, for controls and SLC-samples, respectively). It should be noted that such heavy contamination with A. caviae, or other bacteria, would not normally be present when following strict hygienic semen collection protocols. Presumably, the lower sperm quality was due to this contamination. In the previous study with semen from boars on the same stud as in the present study, S. simulans, K. variicola, E. coli, Enterobacter faecalis and Pseudomonas species were found, albeit in much lower counts, but A. caviae was not cultured from semen [11]. This is a relevant finding, following previous studies on the effects of bacterial contamination on boar semen [4,6,20,21] and highlights the effects that even occasional contamination could produce on sperm quality in boar semen doses. Even though semen collection protocols in the pig industry are very strict, studies have demonstrated that such contamination might occur [4,9,22].

Apart from S. simulans, which is a skin organism, and E. coli and Ent. faecalis, which are found in the gut, the bacterial species identified in the present study are environmental organisms found in soil and water, in accordance with previous studies [11]. The bacteria that live on the body are usually commensals but may cause infection under particular circumstances, such as in contaminated wounds, reduced host immunity or in conjunction with other bacterial infections. Furthermore, enterobacteria and other groups contain antibiotic-resistant strains, and are able to transfer bacterial resistance to less resistant strains [23]. In the present study, their presence was associated with a decrease in membrane integrity, as well as increased capacitation and reacted or damaged acrosomes. Previous studies reported decreased motility in the presence of E coli, possibly by causing sperm aggregation, or may be spermatotoxic [24,25]. Bussalleu et al. [26] reported a significant decline in sperm quality after inoculation of E. coli into sperm doses. Ubeda et al. [20] reported a negative effect

Table 3
Proportion of samples (out of eight) free of specific bacteria, other than Aeromonas.

	day 0			day 3			day 7		
Species	CON	P20	P30	CON	P20	P30	CON	P20	P30
Pseudomonas aeuriginosa	75%	75%	75%	50%	50%	50%	50%	62.5%	562.5%
Staphylococcus simulans	87.5%	100%	100%	62.5%	100%	100%	62.5%	100%	100%
Escherichia coli	37.5%	62.5%	62.5%	37.5%	75%	75%	37.5%	75%	75%
Enterococcus faecalis	62.5%	75%	75%	62.5%	75%	87.5%	62.5%	75%	75%
Bacillus licheniformis	75%	100%	100%	75%	100%	100%	75%	100%	100%
Citrobacter koseri	75%	100%	100%	75%	100%	100%	75%	100%	100%
Proteus vulgaris	87.5%	100%	100%	87.5%	100%	100%	87.5%	100%	100%
Klebsiella variicola	62.5%	62.5%	62.5%	62.5%	62.5%	75%	62.5%	62.5%	62.5%
Total	0%	25%	12.5%	0%	0%	12.5%	0%	0%	0%

Note: CON = Control, P20 and P30 = 20% and 30% Porcicoll, respectively.

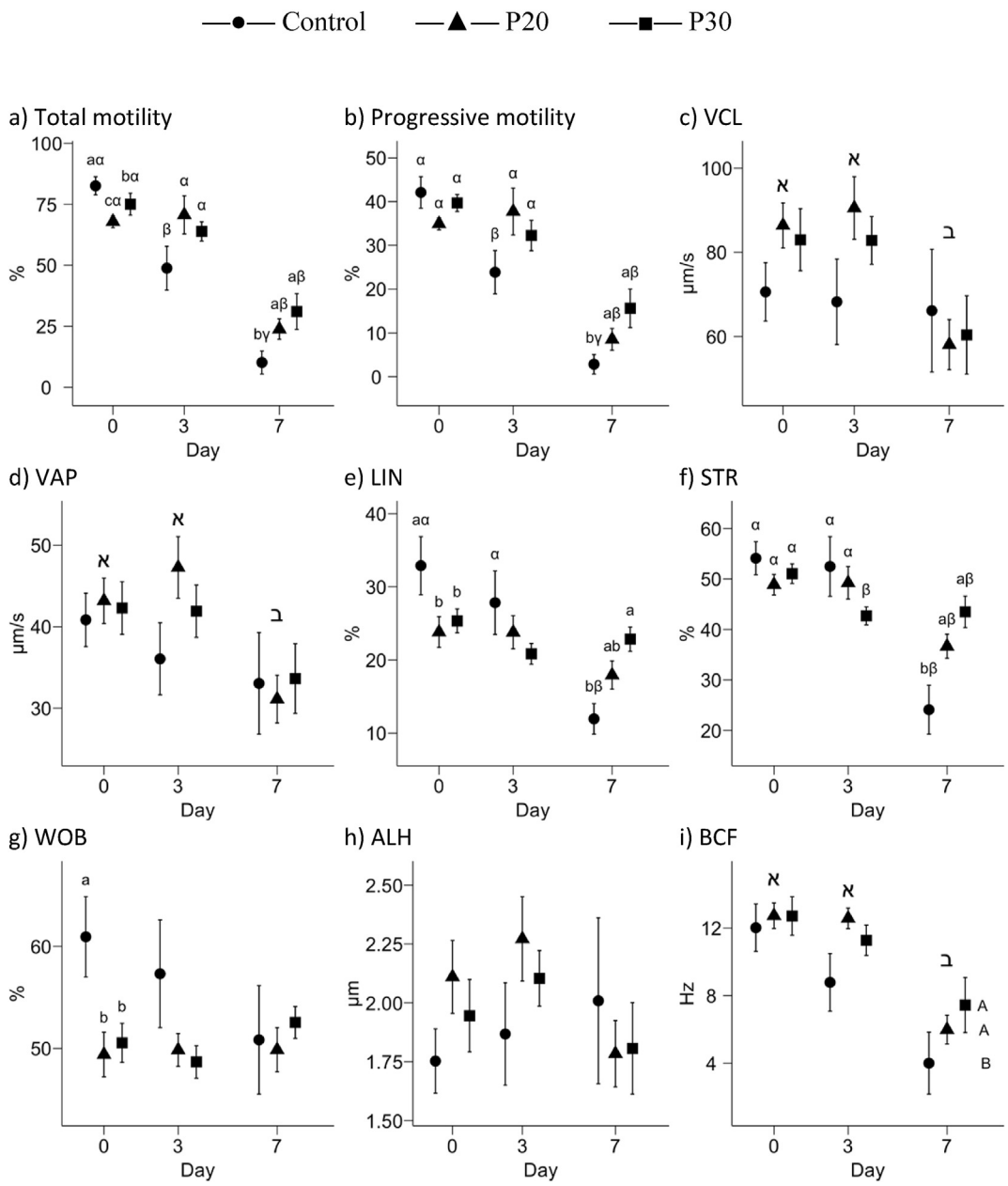

Fig. 2. Effect of colloid centrifugation on average CASA variables (mean ± SEM) for each treatment and storage day.
Interactions between treatments and storage time are shown as follows: different lowercase latin letters indicate $P < 0.05$ among treatments within days, and different greek letters indicate $P < 0.05$ among days within treatments. When the interaction was not significant, the factors were studied as main effects, with different uppercase latin letters showing $P < 0.05$ among treatments, and different hebrew letters showing $P < 0.05$ among days. VCL: Curvilinear velocity; VSL: Straight path velocity; VAP: Average path velocity according to the average smoothed path; µm/s; LIN: Linearity; STR: Straightness; WOB: wobble; ALH: amplitude of the lateral displacement of the sperm head; BCF: Frequency of the flagellar beat.

of S. marcescens, K. oxytoca, M. morganii, and P. mirabilis on sperm quality, but not of E. coli, possibly due to low concentrations or strain differences. Prieto-Martinez et al. [27] showed that increasing bacterial contamination of boar semen with Ent. cloacae was associated with decreased sperm motility, membrane integrity and osmotic resistance, and increased sperm agglutination. Sperm motility, viability and acrosome integrity were reduced when high numbers ($2x10^8$ cfu/mL) of P. aeruginosa were inoculated in boar semen [28,29]. Similar deterioration in sperm quality (decreased motility, membrane integrity and acrosome integrity, as well as increased agglutination) was observed in naturally contaminated boar semen doses used for AI in Poland (most often, Staphylococcus, Streptococcus and Pseudomonas, with some occurrence of enterobacteria and Bacillus), even in the presence of the antibiotic gentamicin [30]. Although the sample size in the present study is small, and therefore possible effects of bacterial contamination must be interpreted with caution, there were several negative correlations of bacterial counts with sperm quality, including environmental bacteria such as Aeromonas. A possible confounder is the likely interaction between the direct effects of the SLC with low density colloid, possibly resulting in retention of some dead spermatozoa together with bacteria. Nevertheless, our results agree with previous studies focused on analysing the effect of bacterial contamination on boar semen [6,24,31–33]. It was

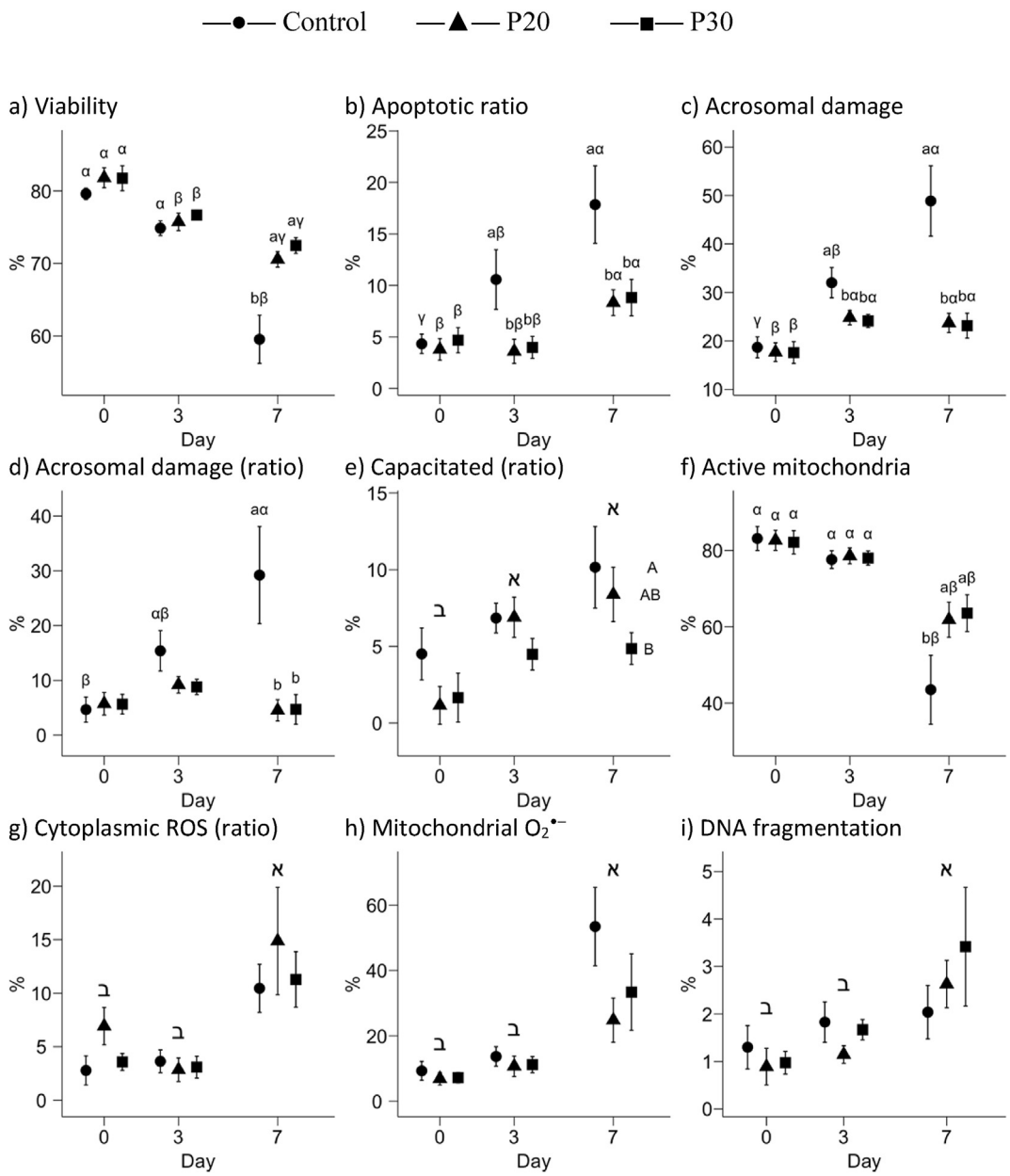

Fig. 3. Effect of the colloid centrifugation on flow cytometry parameters (mean ± SEM) for each combination of treatment and storage day.
Parameters defined as ratios correspond only to the subpopulation considered as viable. When there was an interaction between factors, different lowercase latin letters indicate $P < 0.05$ among treatments within days, and different greek letters indicate $P < 0.05$ among days within treatments. When the interaction was not significant, the factors were studied as main effects, with different uppercase latin letters showing $P < 0.05$ among treatments, and different hebrew letters showing $P < 0.05$ among days.

interesting to note that A. caviae, which was not removed by SLC, showed a strong association with some sperm quality variables. However, it should be noted that this study was designed to test the ability of a large volume SLC with a low density colloid to remove bacteria while recovering a high number of spermatozoa, and therefore the samples were deliberately contaminated with bacteria including A. caviae. This study is a first step in developing a practical protocol for the AI industry. Follow-up studies will assess the suitability of this large volume SLC for producing commercial semen doses, and testing them for AI in production centres.

This scaled-up SLC in 500 mL tubes using a high density colloid was reported previously to be appropriate for processing the voluminous ejaculate of the boar [14,34]. However, the loss of some spermatozoa could be of concern to the breeding industry [34]. The current version with the low density colloid appears to offer a practical solution for removing bacteria from boar semen, and could be an alternative to adding antibiotics. There are many problems with use of antibiotics: apart from being non-therapeutic and therefore going against the recommendations for prudent use of antimicrobials, they can cause antibiotic resistance in the bacteria commonly found in semen. Bacteria were cultured from boar semen doses containing antibiotics [8,21,30]. Thus, bacteria are still present in the sample; their presence may adversely affect sperm quality, as shown here, or may impair fertility in the sow after artificial insemination [6,35]. Finding new antibiotic substances provides a temporary solution but microorganisms are adept at developing resistance to antibiotic substances. Even if the antibiotic is effective against these bacteria, the presence of dead bacteria

Table 4
Spearman correlations between the presence of bacterial species (CFU/mL) and the sperm quality variables analysed. Only associations with P < 0.01 are shown.

Species	Variable	r	P
A. caviae	%DFI	0.348	0.003
	Active mitochondria	−0.341	0.003
	Damaged acrosomes	0.385	0.001
	Mitochondrial ROS (ratio)	0.615	<0.001
	Progressive motility	−0.315	0.007
	Total motility	−0.426	<0.001
	Viability	−0.486	<0.001
C. koseri	Damaged acrosomes	0.305	0.009
Ent. faecalis	%DFI	0.520	<0.001
	Active mitochondria	−0.365	0.002
	Capacitated (ratio)	−0.320	0.006
	Damaged acrosomes	0.466	<0.001
	Mitochondrial ROS (ratio)	0.327	0.005
	Viability	−0.536	<0.001
E. coli	Damaged acrosomes	0.376	0.001
	Viability	−0.413	<0.001
K. variicola	Apoptotic (ratio)	0.317	0.007
	Capacitated (ratio)	0.527	<0.001
	LIN	−0.433	<0.001
	STR	−0.423	<0.001
Total bacteria (excluding Aeromonas)	%DFI	0.362	0.002
	Damaged acrosomes	0.474	<0.001
	Mitochondrial ROS (ratio)	0.335	0.004
	Viability	−0.472	<0.001

%DFI: DNA fragmentation; LIN: Linearity; STR: Straightness.

may also adversely affect sperm quality, e.g. by release of intracellular substances after cell wall disruption, or the release of lipopolysaccharide which gram negative bacteria contain in their cell walls [36]. Physical removal of the bacteria would prevent such adverse effects. Another method of reducing bacterial multiplication is by lowering the storage temperature of the semen to 4 °C. Low temperature extenders, although recently available for pig semen (e.g. Refs. [37,38], have not proved popular with the pig breeding industry. However, even if bacteria do not multiply during refrigeration, they will grow and produce toxic substances during the period before refrigeration temperature is reached, and again between removing the insemination dose from the refrigerator while preparing it for insemination [39]. Thus, they have the potential to affect sperm quality and the inseminated sow [6]. Other methods are based on the addition of unconventional antimicrobial substances, either plant extracts [40,41] or antimicrobial peptides [42]. However, addition of novel antimicrobial agents does not preclude the emergence of bacterial resistance.

A combination of physical removal of most of the bacteria from the semen by low density colloid centrifugation, and low temperature storage to prevent the remaining bacteria from multiplying during storage, would seem to be a possible alternative to antibiotics for the pig breeding industry. Despite the promising results obtained in this study, two aspects must be investigated before putting this methodology into practice. First, the processed doses must prove a similar, if not superior, fertility in AI. Second, between-boar variability should be assessed. Boars produce ejaculates of different intrinsic properties [43], which might affect the yield after SLC and the resilience of the resulting sperm doses. Whereas some information is available from previous studies using SLC with small volumes of colloid [44,45], specific experiments are needed to evaluate this potential between-boar variability.

5. Conclusions

Low density Porcicoll (either 20% or 30%) SLC removed most of the bacteria from heavily contaminated boar semen samples, apart from A. caviae, which would not normally be present in such samples. Scaled-up SLC in 500 mL tubes enabled even large volume pig ejaculates to be processed easily. Sperm quality in the control samples deteriorated more quickly than in the SLC samples during storage for seven days; there was no difference in sperm quality between P20 and P30. These results are very encouraging for the development of an alternative to antibiotics to control bacteria in boar semen samples.

CRediT authorship contribution statement

F. Martínez-Pastor: Conceptualization, Visualization, analysis, Resources, Writing - review & editing, Supervision, Project administration, Resources. **E. Lacalle:** Investigation, Validation, Writing - review & editing. **S. Martínez-Martínez:** Investigation, Methodology, Validation, Formal analysis, Resources, Data curation, Writing - review & editing. **E. Fernández-Alegre:** Investigation, Writing - review & editing. **L. Álvarez-Fernández:** Investigation, Validation, Writing - review & editing. **M.-J. Martinez-Alborcia:** Investigation, Validation, Writing - review & editing. **A. Bolarin:** Conceptualization, Resources, Supervision, Writing - review & editing. **J.M. Morrell:** Conceptualization, Visualization, Methodology, Investigation, analysis, Resources, Writing - original draft, Supervision, Project administration, Resources.

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